Identification and Quantification of Important Steryl Esters in Aspen Wood

Alessio N. Serreqi, Robert Leone, Luis F. Del Rio, Shirley Mei, Marc Fernandez, and Colette Breuil*

Chair of Forest Products Biotechnology, Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z4,

ABSTRACT: Steryl esters make up a major portion of the total lipids in aspen wood, and contribute significantly to pitch deposit problems during pulping. Fungal treatment of aspen is an attractive method for removing these compounds because it is inexpensive and environmentally acceptable; however, the mechanism of steryl ester removal remains unclear. Identification of the steryl esters will lead to a better understanding of how they are removed by fungi. The steryl ester fraction from aspen wood was obtained by acetone extraction then further purified by silica gel column chromatography and argentation-silica gel column chromatography. This led to the isolation of three major fractions: fraction I, fraction II, and fraction III. The major steryl esters of fractions I and II were identified by gas chromatography, gas chromatography–mass spectroscopy, and proton nuclear magnetic resonance analysis of the intact fraction as well as sterol and fatty acid moieties obtained after base hydrolysis. Identification of the steryl esters was carried out by mass spectra comparisons with steryl ester standards synthesized in the laboratory and comparison with mass spectra libraries (Wiley and NIST) by mass fragmentography. Fraction I contained primarily the palmitate, stearate, and eicosanoate esters of α- and β-amyrin. Fraction II consisted mainly of the palmitate, stearate, and eicosanoate esters of tirucalla-7,24-dien-3β-ol and lupeol.

Paper no. J9405 in *JAOCS 77,* 413–418 (April 2000).

KEY WORDS: Aspen, argentation-silica gel chromatography, gas chromatography, gas chromatography–mass spectrometry, pitch analysis, *Populus tremuloides Michx,* sterols, steryl esters, triterpenes.

In aspen-pulping processes, pitch depositions continue to be a serious problem (1,2). Pitch problems are primarily caused by lipophilic wood extractives, commonly called pitch or wood resins, which consist mainly of fatty and resin acids, glycerides, terpene alcohols, sterols, steryl esters, and waxes (1,3). The roles of triglycerides, fatty acids, and resin acids in initiating pitch deposits are well documented, while the role of triterpene alcohols, sterols, steryl esters (SE), and waxes (W) in pitch formation is poorly understood. The low ratio of saponifiables to unsaponifiables, such as sterols, SE, and W in aspen wood, relative to softwoods, has been reported as contributing substantially to the frequent pitch problems during kraft pulping (4). Seasoning of aspen logs or wood chips is currently the method of choice to reduce pitch deposition. For whole logs, usually 12 mon of seasoning is required, while for wood chips in piles, seasoning may take 2 mon. The long storage times and management of the wood make this method tedious and expensive. Also, appreciable loss of brightness and pulp yield is observed due to sap stain and decay fungi.

Treating wood with fungi that consume the lipophilic extractives has been studied as a way of accelerating seasoning and reducing pitch deposition (5–8). Fungal treatment of wood is an attractive method for removing extractives because it is environmentally benign and relatively inexpensive. Recent work on fungal treatment of aspen chips with Cartapip® showed that the fungi did not significantly reduce SE and W (9,10). In complementary work, a series of fungi were tested for their ability to consume the problematic steryl esters and waxes of aspen in liquid media (11). Several fungi were identified that could potentially be used to biotreat aspen wood. To better understand the mechanism of extractive removal by fungal treatment, it is important to identify the individual components of the SE and W.

The free sterols and triterpene alcohols, free fatty acids, combined sterols and triterpene alcohols, and combined fatty acids in SE from aspen wood have previously been identified (12). This analysis, however, gives little information on the composition of the intact SE. To determine the structure of the intact SE of aspen wood, the SE fraction was further resolved by chromatographic techniques. The fractions were analyzed by gas chromatography (GC) and GC–mass spectrometry (GC–MS), and the components were identified by comparison with SE standards synthesized in the laboratory. Further identification of the SE was done by mass spectra comparisons with the SE standards and comparison with mass spectra in the Wiley and NIST libraries.

MATERIALS AND METHODS

Reagents. Analytical thin-layer chromatography (TLC) plates (Whatman silica gel G, F-254, 250-mm layer) were purchased

*To whom correspondence should be addressed at Department of Wood Science, Faculty of Forestry, University of British Columbia, 4041-2424 Main Mall, Vancouver, British Columbia, Canada, V6T 1Z4.
E-mail: breuil@interchg.ubc.ca

from Fisher Scientific (Nepean, ON). Spots were visualized by staining with phosphomolybdic acid in EtOH/H_2SO_4 (4:1) and heating. Column chromatography was carried out on silica gel 200–400 mesh, 60Å purchased from Aldrich (Milwaukee, WI). Silver nitrate and lupeol were purchased from Sigma Chemical Co. (St. Louis, MO); α-amyrin was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). All fatty acid standards were purchased from Aldrich. All solvents used were analytical grade and obtained from Fisher Scientific. All gases used were chromatographic grade and purchased from Praxair (Vancouver, BC).

Synthesis of SE standards. SE were prepared according to the procedure of Hassner and Alexanian (13). In a typical reaction, α-amyrin (4.5 mg, 11 μmol) was dissolved in methylene chloride (1.0 mL) and treated with *N,N′*-dicyclohexylcarbodiimide (9.0 mg, 44 μmol), palmitic acid (16.0 mg, 63 μmol), and catalytic amounts of 4-dimethylaminopyridine (a few crystals). The reaction was stirred at room temperature for 18 h and the resulting ester was purified by solid-phase extraction (SPE) (14) to give a white amorphous solid (5.0 mg, 7.5 μmol, 68% yield). The purity of the synthetic ester was determined by analytical TLC (R_f = 0.86, hexane/EtOAc, 9:1); ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$, 200 MHz) δ 0.78, 0.85, 0.89, 0.96, 0.99, 1.05, 1.23, 2.27 (2H, *t*, *J* = 7 Hz), 4.49 (1H, *m*), 5.10 (1H, *t*, *J* = 3.6 Hz); IR ν_{max}/cm^{-1} 2930, 1740, 1660, 1450, 1380, 1250, 1160; mass spectral data in Table 1.

Lupeol palmitate was synthesized in the same manner as described above. The product was obtained as a white amorphous solid (4.7 mg, 7.1 μmol, 95% yield). The purity of the synthetic ester was determined by analytical TLC (R_f = 0.86, hexane/EtOAc, 9:1); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.77, 0.82, 0.85, 0.92, 1.23, 2.27 (2H, *t*, *J* = 7 Hz), 4.45 (1H, *m*), 4.55 (1H, *br* s), 4.66 (1H, *br* s); IR ν_{max}/cm^{-1} 2924, 1728, 1640, 1468, 1382, 1180; mass spectral data in Table 1.

A list of all prepared SE standards and their mass spectra data are given in Table 1.

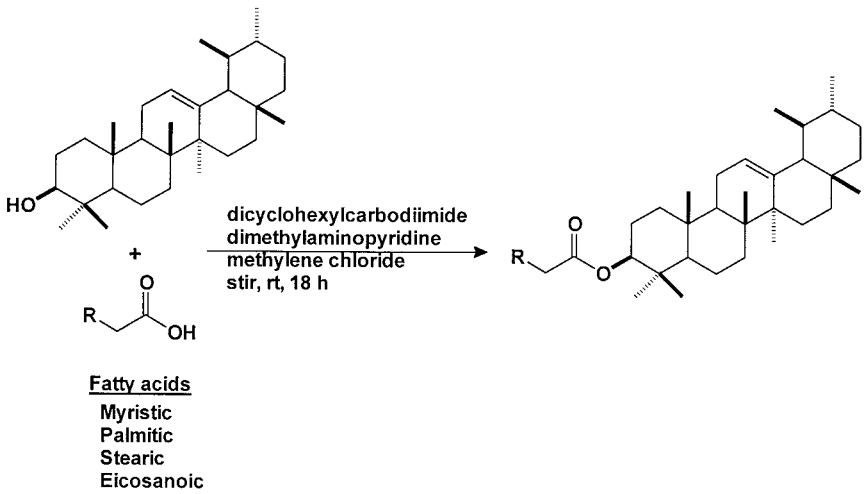
Extraction and fractionation of SE. The aspen wood was from a 68-year-old tree in northern Alberta. The freshly cut wood was reduced to small blocks and immediately frozen at –20°C. The moisture content of the wood, determined gravimetrically after drying at 105°C overnight, was 28.4%. The wood chips were ground to fine sawdust powder before acetone extraction in a Soxhlet apparatus (18 h, 5–6 cycles/h). After extraction, the acetone was dried over magnesium sulfate, filtered, then concentrated by rotary evaporation and dried under vacuum to give the acetone extractives of aspen [54 ± 1 g/kg oven-dried (OD) wood]. The SE/W fraction was obtained by applying the total extractives on a silica gel column eluted with hexane/ether (50:1). The purified fractions were pooled, concentrated, and vacuum-dried to yield the SE/W (3.1 ± 0.3 g/kg OD wood). The SE/W fraction was further purified by 10% argentation-silica gel column chromatography (~0.4 g/mg of residue) eluted with a hexane/toluene gradient from 4:1 to 1:4, then 100% ethyl acetate. The components were separated by their polarity and degree of saturation into three fractions referred to as fractions I, II, and III.

Fractionation of fraction II. An analytical TLC plate was impregnated with 2% silver nitrate in methanol then air dried and activated in an oven at 105°C for approximately 15 min. Five milligrams of fraction II dissolved in 0.5 mL of ethyl ether was applied to the plate with a glass pipette. The plate was then eluted with 60:40 hexane/toluene in a large development tank. Visualization was carried out by cutting a strip from the plate and staining with phosphomolybdic acid. The two major bands observed on the strip were used to locate the bands on the plate. The bands were scraped off and extracted with ethyl ether (3 × 3 mL). The solutions were filtered into vials and dried under a stream of N_2. The samples were further purified on a short silica gel column eluted with 80:20 hexane/toluene and dried under N_2, then under vacuum to give fraction II 1 and 2.

Hydrolysis of steryl esters. The eluted SE (15–25 mg) were refluxed in 0.5 N potassium hydroxide in 90% ethanol (5 mL) until the reaction was complete (5–6 h), as verified by TLC. The solutions were then acidified to pH 2.0 with 1 N HCl and concentrated by rotary evaporation at 50°C. The saponified residues were dissolved in chloroform and run through an anhydrous magnesium sulfate column. The solutions were then dried under a stream of N_2 gas and fractionated by SPE according to the method of Chen *et al.* (14) to give a sterol fraction and a fatty acid fraction.

Detection of the components in the SE fraction by GC. The Hewlett-Packard (Palo Alto, CA) HP 5890 Series II gas chromatograph was equipped with a flame-ionization detector (FID) and an HP 7673 automatic sampler. The SE were separated on a DB-5 fused-silica capillary column (15 m × 0.25 mm i.d., 0.25-μm film, J&W Scientific, Folsom, CA). The temperature program started at 50°C, increased at 10°C/min to 325°C, and then was held for 45 min. The total running time was 65 min. Helium was used as the carrier gas at a flow rate of 30 cm/s. The injection volume was 2.0 μL. The inlet temperature was set at 330°C and the FID at 330°C. All the samples analyzed by GC were injected at a concentration of 1.0 mg/mL. The equipment, data collection, and processing were controlled by Hewlett-Packard HP 3365 Series II software. Relative retention times (RRT) and concentrations of GC peaks were calculated relative to the internal standard, cholesteryl myristate, which was added before GC analysis. A response factor of 0.64 was determined for cholesteryl myristate against α-amyrin palmitate. This value was used to determine the component concentrations in fraction I. A response factor of 0.76 was determined against lupeol palmitate and this value was used in determining concentrations of fraction II components.

Detection of components in SE fraction by GC–MS. A Varian (Palo Alto) 3800 GC was coupled to a Varian Saturn 2000 MS (ion trap detector). The GC was equipped with a J&W DB-XLBitd column (10 m × 0.25 mm diameter, 0.25-m film thickness), and a deactivated 1078 fritted splitter inlet sleeve loaded on a 1079 injector. The MS electron multiplier voltage was set at 2,150 V, with an ionization time of 25,000 μs, running in electron ionization (EI) mode, with transfer line,



SCHEME 1

trap, and manifold temperatures of 350, 250, and 50°C, respectively. Samples were injected *via* a Varian 8200 autosampler fitted with a 10-µL syringe. The SE/W fractions and the synthetic SE standards were dissolved in hexane or chloroform at various concentrations. Subsequently, 1–5 µL of these solutions was injected onto the GC–MS with a split of 1:60, and injector temperature of 300°C. A continuous flow rate of 1.6 mL/min of helium was used with a temperature profile starting at 200°C for 1 min followed by a 15°C/min ramp to 300°C, then finally a 2°C /min ramp to 325°C which was held for 48 min.

Identification of SE. The SE from the three fractions of aspen wood were identified by mass spectra comparison with those in the NIST and Wiley libraries and, when possible, by comparisons of mass spectra of standards.

RESULTS AND DISCUSSION

Synthesis of SE standards. A series of standard SE was synthesized by combining commercially available sterols and triterpenes with the major fatty acids using dicyclohexylcarbodiimide as a coupling agent (Scheme 1, Table 1). These standards were then used to identify the intact SE through both spiking experiments and by comparing their GC–MS fragmentation patterns. This approach was necessary because the mass of most SE was greater than the maximum detectable mass of the GC–MS system.

Purification and identification of SE in aspen wood. After Soxhlet extraction of ground aspen wood with acetone, medium-pressure silica gel column chromatography was used to separate the wood extractives based on the component po-

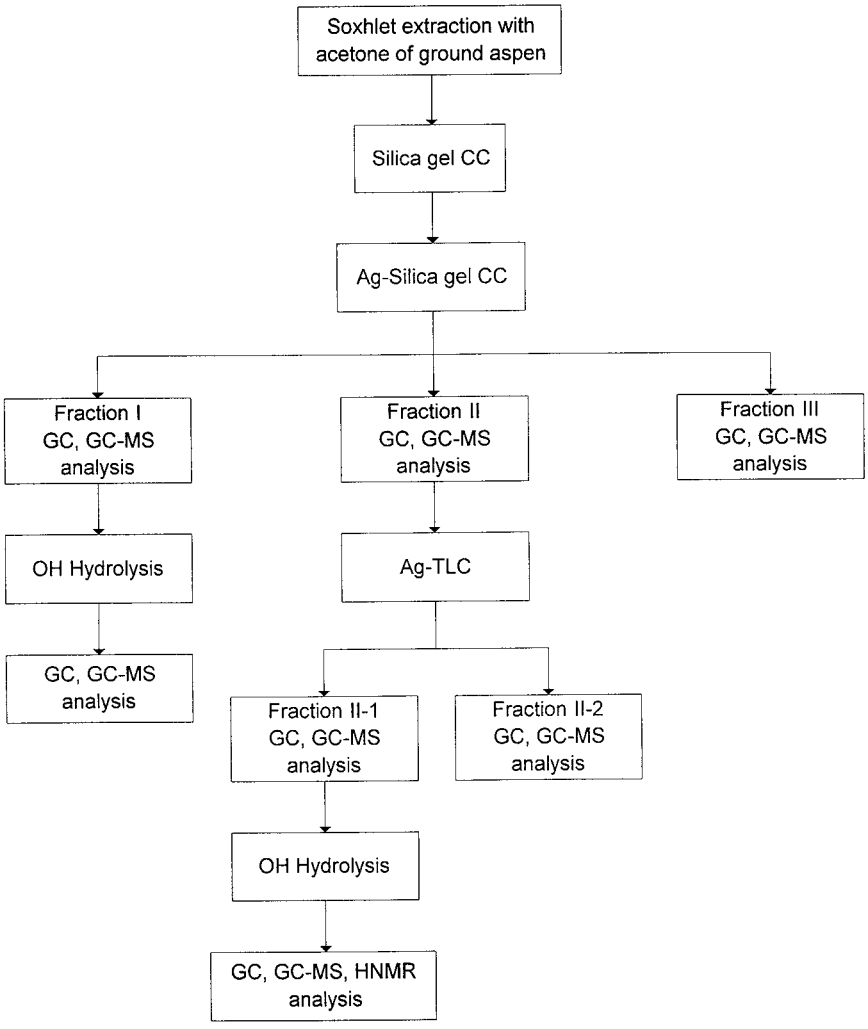
TABLE 1
Electron Impact (70 eV) Mass Spectra Profiles of Synthesized Steryl Ester Standards

Steryl ester	Molecular weight	Mass spectral data (% relative intensity)
α-Amyrin myristate	636	55(23), 69(20), 81(22), 95(35), 107(30), 122(40), 135(38), 147(52), 161(41), 175(24), 189(87), 203(68), 218(100), 231(10), 270(12), 409(11)
α-Amyrin palmitate	664	55(16), 69(16), 81(18), 95(29), 107(23), 122(33), 135(35), 147(38), 161(38), 175(21), 189(79), 203(64), 218(100), 231(11), 270(12), 409(10)
α-Amyrin-stearate	692	55(22), 69(18), 81(21), 95(29), 107(28), 122(35), 135(33), 147(45), 161(38), 176(22), 190(30), 203(23), 218(100), 231(11), 271(8), 410(11)
α-Amyrin eicosanoate	720	55(24), 69(22), 81(18), 95(29), 107(28), 121(34), 135(30), 147(39), 161(39), 17622(22), 190(28), 204(68), 218(100), 231(10), 271(9), 410(11)
Lupeol myristate	636	55(23), 67(34), 81(34), 95(54), 109(44), 121(42), 135(37), 147(31), 161(31), 175(32), 189(100), 203(43), 216(20), 229(17), 257(9), 297(14), 393(14), 410(12)
Lupeol palmitate	664	55(23), 57(23), 67(32), 81(35), 95(56), 109(42), 121(45), 135(37), 147(33), 161(34), 175(34), 189(100), 203(49), 216(22), 229(18), 257(10), 297(12), 410(14)
Lupeol stearate	692	55(19), 57(19), 67(26), 81(35), 95(46), 109(36), 121(38), 135(16), 147(33), 161(30), 175(26), 189(100), 203(49), 216(20), 229(16), 257(11), 297(14), 394(14), 410(12)
Lupeol eicosanoate	720	55(22), 57(21), 67(34), 81(34), 95(55), 109(41), 121(42), 135(35), 147(34), 161(32), 175(31), 189(100), 203(24), 216(20), 229(16), 257(11), 297(12), 394(15), 410(14)

larities. The nonpolar SE were eluted with a hexane/ether mixture and recovered in the first several fractions. The SE were then purified further by argentation-silica gel chromatography, which separated, on the basis of their polarity and degree and mode of unsaturation, into three fractions: fractions I, II, III (Scheme 2). Fraction I was base-hydrolyzed and the resulting sterols and fatty acids were analyzed by GC–MS according to the method of Peng *et al.* (12). The primary fatty acids were identified as palmitic, stearic, and eicosanoic acid, while the main triterpenes were α- and β-amyrin. From this information, the α-amyrin ester standards were synthesized and compared to the components in unhydrolyzed fraction I. Through GC spiking experiments and comparison of the mass spectra fragmentation data, the primary α-amyrin esters in fraction I were identified. The very similar mass spectra fragmentation pattern of the other components led to their β-amyrin ester assignment.

Fraction II was analyzed in the same manner as described above. The primary fatty acids were identified as palmitic, stearic, and eicosanoic acids, while the main triterpenes were lupeol and an unidentified compound similar to lanosterol. The structure of the unknown triterpene was determined through further purification of fraction II by argentation thin-layer chromatography. This led to the isolation of two subfractions: fraction II-1 and fraction II-2. Fraction II-1 was base-hydrolyzed and the resulting triterpene alcohol was isolated and analyzed by proton NMR and identified as tirucalla-7,24-dien-3β-ol by comparison to literature data (15). Lupeol ester standards were synthesized and compared to the components in unhydrolyzed fraction II. Through GC spiking experiments and comparison of the mass spectra data, the primary lupeol esters in fraction II were identified.

Concentration and composition of SE in aspen wood. In aspen wood, the SE/W were present at 3.1 ± 0.3 g/kg OD wood. Fraction I accounted for 28 ± 2% of the total SE/W fraction. The eight major identified components of this fraction represented 67% of the material balance (Table 2). The palmitate esters were the most abundant and α-amyrin palmitate alone accounted for 33% of this fraction. Fraction II represents 35 ± 7% of the total SE/W fraction. From this frac-



SCHEME 2

TABLE 2
Steryl Esters Present in Fraction I

Steryl ester	RR_t[a]	Composition (%) of total steryl esters	Content (mg/kg freeze-dried wood) in fraction I
β-Amyrin myristate	1.11	0.08	3
α-Amyrin myristate	1.15	0.2	5
β-Amyrin palmitate	1.29	5.6	170
α-Amyrin palmitate	1.34	9.5	290
β-Amyrin stearate	1.50	1.0	29
α-Amyrin stearate	1.56	1.6	48
β-Amyrin eicosanoate	1.80	0.4	12
α-Amyrin eicosanoate	1.89	0.7	22

[a]Relative retention times (RR_t) of steryl esters on DB5 capillary column (cholesteryl myristate: 1.00).

TABLE 3
Steryl Esters Present in Fraction II-1 and Fraction II-2

Steryl ester	RR_t[a]	Composition (%) of total steryl esters	Content (mg/kg freeze-dried wood) in fraction I
Tirucalla-7,24-dien-3β-yl palmitate	1.26	4.2	129
Lupeol palmitate	1.33	11.1	338
Tirucalla-7,24-dien-3β-yl stearate	1.49	0.9	27
Lupeol stearate	1.55	2.8	85
Tirucalla-7,24-dien-3β-yl eicosanoate	1.75	0.2	5
Lupeol eicosanoate	1.87	1.0	30

[a]RR_t of steryl esters on DB5 capillary column (cholesteryl myristate: 1.00). See Table 2 for abbreviation.

tion, six components were identified that made up 61% of the material (Table 3). Again, the palmitate esters were the most abundant, and lupeol palmitate accounted for 33% of the fraction. It also was the most abundant SE present in aspen, accounting for 11% of the total.

Fraction III represents 37 ± 8% of the total SE/W fraction. At present, the components in this fraction appear to be primarily steryl linoleate esters. Unfortunately, GC analysis of this fraction gave a complex mixture of unresolved and broad peaks. Therefore, more research is needed to conclusively identify the SE in this fraction. The components that have been identified and quantified account for 1.2 g/kg OD wood or 39% of the SE/W fraction. The identification of SE in aspen should enable a better understanding of the mechanism of their removal during seasoning or biotreatment.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science and Engineering Research Council. The authors would like to thank Drs. Paul Watson and Bruce Sitholé of PAPRICAN for their valuable advice.

REFERENCES

1. Allen, L.H., B.B. Sitholé, J.M. Macleod, C.L. Lapointe, and F.J. McPhee, The Importance of Seasoning and Barking in the Kraft Pulping of Aspen, *J. Pulp Paper Sci. 17*:J85–J91 (1991).
2. Chen, T., Z. Wang, Y. Zhou, C. Breuil, O.K. Aschim, E. Yee, and L. Nadeau, Using Solid-Phase Extraction to Assess Why Aspen Causes More Pitch Problems Than Softwoods in Kraft Pulping, *Tappi J. 78*:143–149 (1995).
3. Sjöström, E., *Wood Chemistry: Fundamentals and Applications*, Academic Press Inc., San Diego, 1993, pp. 90–113.
4. Sitholé, B.B., J.L. Sullivan, and L.H. Allen, Identification and Quantification of Acetone Extractives of Wood and Bark by Ion Exchange and Capillary GC with a Spreadsheet Program, *Holzforsch. 46*:409–416 (1992).
5. Blanchette, R.A., R.L. Farrell, T.A. Burnes, P.A. Wendler, W. Zimmerman, T.S. Brush, and R.A. Snyder, Biological Control of Pitch in Pulp and Paper Production by *Ophiostoma piliferum*, *Tappi J. 75*:102–106 (1992).
6. Brush, T.S., R.L. Farrell, and C. Ho, Biodegradation of Wood Extractives from Southern Yellow Pine by *Ophiostoma piliferum*, *Ibid. 77*:155–159 (1994).
7. Fisher, K., M. Akhtar, R.A. Blanchette, T.A. Burnes, K. Messner, and T.K. Kirk, Reduction of Resin Content in Wood Chips During Experimental Biological Pulping Processes, *Holzforsch. 48*:285–290 (1994).
8. Chen, T., Z. Wang, Y. Gao, C. Breuil, and J.V. Hatton, Wood Extractives and Pitch Problems: Analysis and Partial Removal by Biological Treatment, *Appita 47*:463–466 (1994).
9. Rocheleau, M.J., B.B. Sitholé, L.H. Allen, S. Iverson, R. Farrell, and Y. Noel, Fungal Treatment of Aspen Chips for Wood Resin Reduction: A Laboratory Evaluation. *J. Pulp Paper Sci. 24*:37–42 (1998).
10. Rocheleau, M.J., B.B. Sitholé, L.H. Allen, and Y. Noel, Fungal Treatment of Aspen Chips for Wood Resin Reduction: Effect on Aged Aspen Wood Chips at Room Temperature and at 5°C, *Holzforsch. 53*:16–20 (1999).
11. Leone, R., and C. Breuil, Filamentous Fungi Can Degrade Aspen Steryl Esters and Waxes, *Intl. Biodet. Biodeg. 41*:133–137 (1998).

12. Peng, J., R. Leone, A.N. Serreqi, and C. Breuil, Are Aspen Sterols and Steryl Esters Changed Structurally by Kraft Pulping and Bleaching? *Tappi J. 82*:204–211 (1999).
13. Hassner, A., and V. Alexanian, Direct Room Temperature Esterification of Carboxylic Acids, *Tetrahedron Lett. 46*: 4475–4478 (1978).
14. Chen, T., C. Breuil, S. Carrière, and J.V. Hatton, Solid-Phase Extraction Can Rapidly Separate Lipid Classes from Acetone Extracts of Wood and Pulp, *Tappi J. 77*:235–240 (1994).
15. Itoh, T., T. Tamura, and T. Matsumoto, Proton Magnetic Resonance Spectra of Tetracyclic Triterpene Alcohols Under the Influence of a Lanthanide Shift Reagent, *Steroids 27*:275–285 (1976).

[Received September 24, 1999; accepted January 25, 2000]